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Smad3 Mediates Activin-Induced Transcription of Follicle-Stimulating Hormone β-Subunit Gene

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Synthesis of FSH by the anterior pituitary is regulated by activin, a member of the TGFβ superfamily of ligands. Activin signals through a pathway that involves the activation of the transcriptional coregulators Smad2 and Smad3. Previous work from our laboratory demonstrated that Smad3, and not Smad2, is sufficient for stimulation of the rat FSHβ promoter in a pituitary-derived cell line LβT2. Here, we used RNA interference technology to independently decrease the expression of Smad proteins in LβT2 cells to further investigate Smad2 and Smad3 roles in activin-dependent regulation of the FSHβ promoter. Down-regulation of Smad2 protein by small interfering RNA duplexes affects only basal transcription of FSHβ, whereas decreased expression of Smad3 abrogates activin-mediated stimulation of FSHβ transcription.

Although highly related, Smad2 and Smad3 differ in their Mad homolog (MH) 1 domains, where the Smad2 protein contains two additional stretches of amino acids that prevent this factor from binding to DNA. We investigated whether these structural features contribute to differential FSHβ transactivation by Smad2 and Smad3. A variety of Smad chimera constructs were generated and used in transient transfection studies to address this question. Only cotransfection of chimera constructs that contain the MH1 domain of Smad3 results in activin-mediated stimulation of the rat FSHβ promoter. Furthermore, the insertion of Smad2 loops into Smad3 protein renders it inactive, suggesting that DNA binding is necessary for Smad3-mediated stimulation of the rat FSHβ promoter. Taken together, these results indicate that the functional differences between Smad2 and Smad3 in their ability to transactivate the rat FSHβ promoter lie primarily within the MH1 domain and involve structural motifs that affect DNA binding. (*Molecular Endocrinology* 19: 1849–1858, 2005)

THE ACTIVIN PROTEINS are growth factors that control a variety of cellular and physiological functions including hormone production, cell growth, differentiation, and survival, as well as developmental processes (1–3). Activins are disulfide-linked homo- and heterodimeric species of two homologous β-subunit chains, βA and βB. Specific subunit dimerization results in three major activin isoforms, designated activin A (βAβA), activin B (βBβB), and activin AB (βAβB) (4, 5). Activin is a member of the TGFβ superfamily of proteins and it signals through a heteromeric receptor complex and intracellular signaling molecules known as Smad proteins. Signaling begins upon binding of the ligand to the specific type II serine/threonine kinase receptor (ActRII or ActRIIB), which complexes with and phosphorylates the type I receptor [activin receptor-like kinase 4 (ALK4)]. In turn, the activated type I serine/threonine kinase phosphorylates the cytoplasmic coactivating factors, Smads. Phosphorylation of receptor-regulated Smads (Smad2 and Smad3) allows their association with a common-mediator Smad (Smad4) and translocation into the nucleus. Once in the nucleus, the Smad complex associates with tissue- and cell-specific binding proteins (transcription factors, coactivators and corepressors) and regulates the transcription of target genes (reviewed in Refs. 6 and 7).

As a part of the reproductive cycle, activins exert autocrine and paracrine control in the gonads and the pituitary (8). At the level of the pituitary, activin stimulates FSH transcription and secretion (9, 10), a glycoprotein that is an integral component of the mammalian hypothalamic-pituitary-gonadal axis. There are two distinct phases of increased FSH levels during the female reproductive cycle that are necessary for ovulation and follicle selection. The primary surge of FSH inhibits the production of gonadal steroids and stimulates terminal differentiation of the preovulatory or dominant follicle, whereas the secondary surge recruits a new cohort of follicles to ovulate in the next cycle.

It has been hypothesized that the regulatory action of activin on FSH is through transcriptional stimulation

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Abbreviations: ALK, Activin receptor-like kinase; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; MH, Mad homolog; RLUs, relative light units; siRNA, small interfering RNA.

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of the unique FSHβ-subunit at the level of pituitary gonadotropes (11, 12). Our own studies have shown that the rat FSHβ promoter is specifically transactivated by activin in the mouse pituitary gonadotrope-derived cell line LβT2, suggesting that activin is a major regulatory factor of FSH. In fact, it has been demonstrated that activin-mediated regulation of FSHβ gene transcription involves a proximal promoter region that binds both Smad3 and Smad4 proteins (10, 13). Interestingly, Smad3, and not the highly homologous Smad2, was identified as a transcription factor sufficient for stimulation of the FSHβ-subunit in both activin-dependent and independent manners. Like many transcription factors, the Smad proteins have structural domains that account for the different functions of the molecule. The N-terminal portion of the protein, known as the Mad homolog (MH) 1 domain, is important for both DNA binding and Smad-cofactor interactions. The MH2 domain is located in the C-terminal region of the protein and is important for receptor-Smad, Smad-Smad, and Smad-cofactor interactions (reviewed in Refs. 6, 7, and 14).

Although Smad2 and Smad3 share a high sequence homology, they have significant structural and functional differences. The N-terminal domain of Smad3 contains a β-hairpin loop, and it is through this structural domain that this factor directly interacts with DNA elements (15–19). Although Smad2 also contains this hairpin loop sequence, it does not appear to bind DNA. In fact, Smad2 requires assembly with other factors to direct gene transcription, whereas Smad3 can independently act as a DNA-binding transcription factor. Two surface exposed stretches of amino acids that are present in Smad2, and not in Smad3, may be responsible for the main transcriptional differences between these two proteins because they can bury the DNA-binding β-hairpin loop structure and prevent Smad2 from binding its cognate DNA (20). These results suggest that Smad2 and Smad3 may have different subsets of target genes as well as distinct responses upon ligand activation.

Smad2 and Smad3 have unique transcriptional properties based on their ability to bind DNA directly as well as on their cooperation with other transcription factors. Smad2, together with the forkhead domain protein (FAST2), is required for induction of the mouse goosecoid (*gsc*) promoter, whereas Smad3 suppresses this promoter (21). The role of Smad2 and Smad3 in regulation of FSHβ transcription is controversial (22, 23). Our work suggests that Smad3, but not Smad2, regulates the rat FSHβ-subunit promoter activity. Here, we investigate structural differences between Smad2 and Smad3 as the basis for this divergent control of FSHβ gene transcription by these transcription factors. Through a series of transient transfection studies, the most N-terminal domain of Smad3 was identified as necessary for transcriptional activation of the rat FSHβ promoter. We also employed RNA interference technology to confirm our previous findings that Smad3 is both sufficient and necessary for activin-mediated FSHβ transcription, whereas Smad2 may be involved in maintaining of basal FSHβ transcription.

RESULTS

Smad3 Is Involved in Activin-Mediated Regulation of the FSHβ Promoter

We, and others, have previously demonstrated that Smad3, but not the closely related Smad2, is sufficient for transcriptional regulation of the FSHβ-subunit in LβT2 cells. Furthermore, Smad3 synergizes with Smad4 in activin-mediated stimulation of the FSHβ promoter (10, 13). Here, we investigated whether endogenous Smad3 is necessary for basal and activin-induced activation of the rat FSHβ promoter in LβT2 cells. To address this question, RNA interference technology was employed. Specifically, we used small interfering RNA (siRNA) oligoduplexes to suppress expression of Smad2 and Smad3 proteins independently. A stable cell line was generated and used in this experiment. LβT2 cells were transfected with the −338rFSHβ-Luc promoter construct and a stable population of cells (designated hereafter LβT2-F338) was selected using G418 sulfate. Control, Smad2-, or Smad3-specific siRNA duplexes were delivered into the LβT2-F338 cell line, and the cells were then treated with control media or activin for 24 h (Fig. 1). To confirm specificity of the siRNA duplexes, lysates from wells adjacent to those used for the luciferase assay was collected and used for immunoblot analysis (Fig. 1A). As expected, there was a selective down-regulation of endogenous Smad protein expression with the introduction of Smad2- or Smad3-directed siRNA. The results of the luciferase assay show that down-regulation of Smad3, and not Smad2, reduced activin-mediated induction of the rat FSHβ promoter from 9.5-fold in mock-transfected or control siRNA transfected cells to approximately 2.8-fold in Smad3 siRNA transfected samples. Interestingly, a small but significant decrease in the promoter stimulation was observed with Smad2-specific siRNA (Fig. 1B). This result, however, might be due to a decreased basal transcription of FSHβ-subunit, given that activin-mediated stimulation of the promoter does not change with Smad2 siRNA transfection (Fig. 1C).

Activin Induces Smad2 Phosphorylation and Nuclear Translocation in a Gonadotrope-Derived Cell Line

Smad2 and Smad3 are activin receptor-specific cytoplasmic factors that associate with Smad4 to regulate target gene transcription. To investigate whether the inability of Smad2 to transactivate the FSHβ promoter is due to the lack of activin-specific activation of this factor, we examined activin-dependent phosphorylation and nuclear translocation of Smad2. LβT2 cells

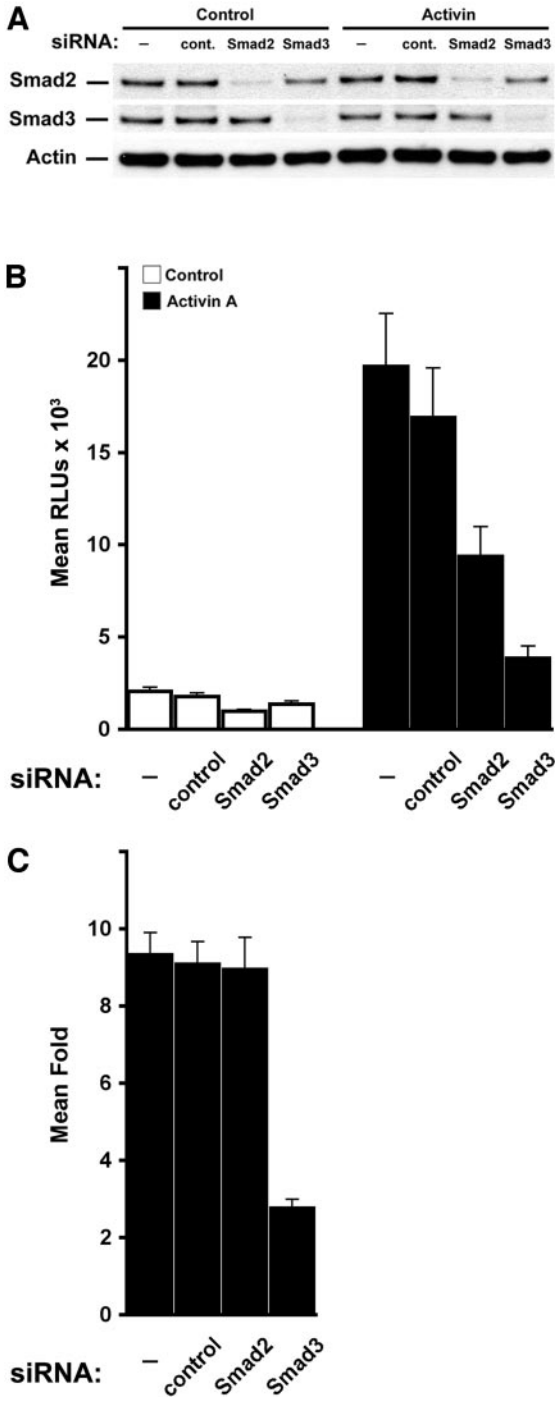

Fig. 1. Down-Regulation of Smad3, But Not Smad2, Protein Expression Affects Activin-Mediated Stimulation of the Rat FSHβ Promoter

LβT2-F338 cells were transfected with indicated Smad siRNA duplexes and treated with control media or activin (30 ng/ml) for 24 h. A, Cells were lysed and cell lysates from one well were run on a polyacrylamide gel. Immunoblot analysis was carried out using anti-Smad2, anti-Smad3 and antiactin antibodies. B, Luciferase assays were performed on cell lysates. Expression of FSHβ is reported as RLUs and plotted as the mean ± SEM of five independent experiments, each performed in triplicate. C, Fold increase of activin-stimulated LβT2-F338 cells transiently transfected with indicated Smad siRNA duplexes. Data are plotted as the mean fold ± SEM of five independent experiments, each performed in triplicate.

were treated with activin for the indicated times and immunoblot of cell lysates was performed using a phosphoSmad2-specific antibody (Fig. 2A). The phosphorylated Smad2 protein can be detected as early as 10 min after activin treatment and the level of phosphorylation increases with time. To determine whether activated Smad2 molecule can translocate into the nucleus, we performed immunohistochemistry to detect phosphorylated Smad2 in LβT2 cells after activin treatment (Fig. 2B). Nuclear accumulation of phosphorylated Smad2 protein is observed within 15 min as seen by its colocalization with 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei of LβT2 cells.

To further investigate whether Smad2 is functional in LβT2 cells, we used an established activin/TGFβ-responsive promoter, the plasminogen activator inhibitor-1, ligated upstream of the luciferase reporter gene (p3TP-Lux) (18). The p3TP-Lux promoter and Smad2 expression vectors were cotransfected into LβT2 cells. Because LβT2 cells do not express TGFβ type II receptor, TβRII, an expression construct encoding the cDNA of this receptor was cotransfected to allow for analysis of the TGFβ response. After transient transfection, cells were treated with activin or TGFβ1 for 24 h. The results show that TGFβ and activin stimulation of the p3TP promoter is further augmented by overexpression of Smad2 (Fig. 2C). This suggests that Smad2 is not only phosphorylated and translocated to the nucleus in the LβT2 cell line, but it is also functional as it can significantly stimulate the p3TP promoter.

MH1 Domain of Smad3 Is Important for Regulation of the Rat FSHβ Promoter

Because Smad2 has only a minimal effect on the FSHβ promoter (10, 13), further studies were carried out to investigate structural domains that may contribute to the differences between Smad2 and Smad3. Toward this end, we investigated the effects of various Smad2/3 chimera constructs on the −338rFSHβ-Luc reporter in LβT2 cells. LβT2 protein expression of all transfected wild-type and chimeric Smad constructs in LβT2 cells was confirmed by immunoblot analysis (Fig. 3). As predicted, cotransfection of the Smad3, but not Smad2, expression vector led to a significant upregulation of FSHβ promoter activity when treated with activin. Interestingly, only cotransfection of chimera constructs that contain the MH1 domain of Smad3 resulted in an additional increase of luciferase expression upon activin treatment (Fig. 4A). Similar results were observed when the rat FSHβ promoter was cotransfected with the constitutively active type I activin receptor (ALK4-CA) expression vector (Fig. 4B).

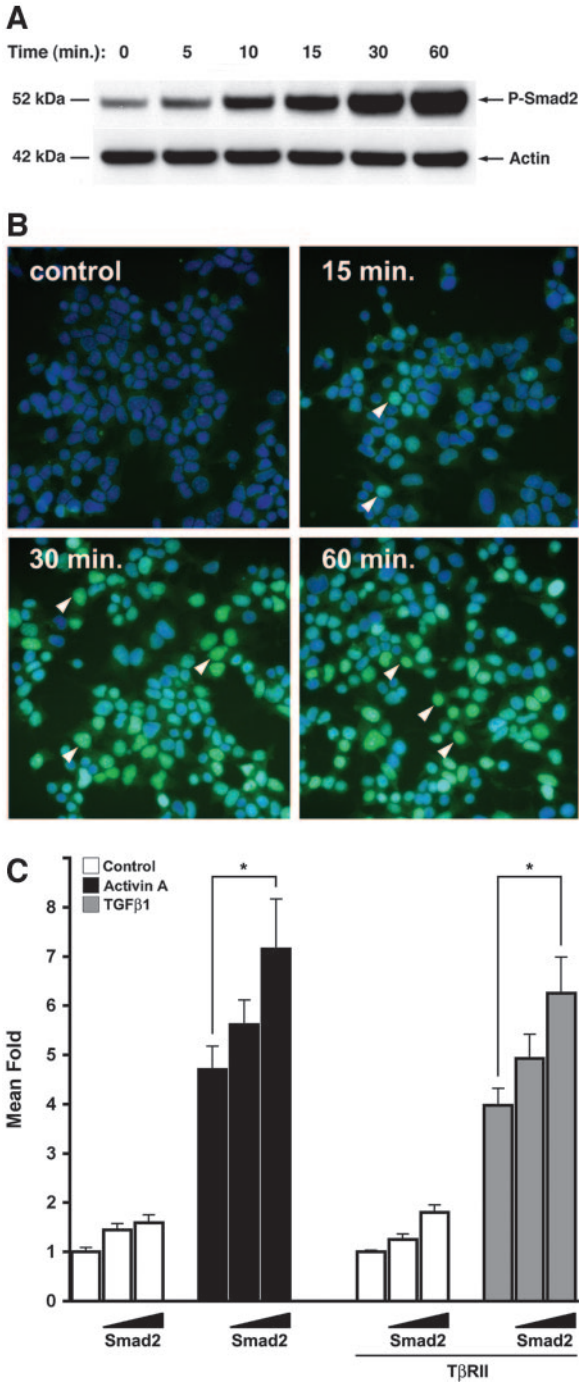

Fig. 2. Smad2 Is Phosphorylated and Functional in LβT2 Cells

LβT2 cells were treated with 30 ng/ml of activin for the indicated times. A, Cell lysates were resolved on a polyacrylamide gel and an immunoblot was performed using the antiphosphoSmad2 rabbit antiserum; the blot was stripped and reprobed with antiactin antibody as a control. B, PhosphoSmad2 protein was detected by immunofluorescence in LβT2 cells using an antiphosphoSmad2 rabbit antiserum and FITC-conjugated antirabbit secondary antibody. Cells were counterstained with DAPI to localize the nucleus and images were taken at ×40 magnification. Colocalization of the FITC and DAPI signals is *aquamarine/light green* and indicated by *arrowheads*. C, LβT2 cells were transiently cotransfected with the p3TP-Lux promoter construct, TβRII (TGFβ1-treated samples only) and increasing amounts of Smad2 expression vector. Cells were treated with control media, activin (30 ng/ml) or TGFβ1 (10 ng/ml) for 24 h. Data are plotted as the mean fold ± SEM of three independent experiments, each performed in quadruplicate. A statistically significant difference between activin alone and Smad2 plus activin samples is denoted by *asterisk* (*, $P < 0.05$).

The N Terminus of Smad3 Is Necessary for Transactivation of the Rat FSHβ Promoter

There are significant structural differences within the MH1 domain of Smad2 and Smad3, particularly in two stretches, or loops, of amino acids present only in Smad2 (Fig. 5A). To further identify the region responsible for the functional differences between these two molecules, we used an additional Smad2/3 chimera construct in which the 5′end of the Smad2 cDNA was exchanged for Smad3 sequence that encodes the first 68 amino acids (S3-MH1N). This plasmid was devoid of both original Smad2 loops. In addition, a naturally occurring Smad2 splice variant, which lacks encoding the second structural loop exon 3 (Smad2Δexon3) and is present in mouse pituitary and LβT2 cells (Fig. 5B), was used. LβT2 cells were transiently transfected with the rat FSHβ promoter and cotransfected with the indicated Smad expression vectors, then the cells were treated with activin for 24 h (Fig. 5C). The chimeric Smad2/Smad3 mutant mimics the synergistic effect of the wild-type Smad3 and activin, suggesting that the important region within the MH1 domain of Smad3 can be narrowed to the first 68 amino acids. Interestingly, although Smad2Δexon3 transactivates the rat FSHβ promoter, this stimulation is 25% lower than wild-type Smad3 or chimeric Smad2/3. This result suggests that the removal of the second structural loop alone is not sufficient for full transcriptional activity of Smad2 and does not fully change its phenotype to that of Smad3.

Structural Motifs Involved in Smad-DNA Interactions Are Important for Smad3-Mediated Activation of the FSHβ Promoter

The N-terminal domain of Smad3 contains an exposed β-hairpin loop that is required for DNA binding. Although Smad2 also contains this sequence, it does not appear to bind DNA and it has been hypothesized that the two Smad2-specific loops hinder the ability of this factor to do so. To investigate whether the Smad2-like loops can affect the ability of Smad3 to transactivate the FSHβ promoter, a series of mutant constructs that have either one or both loops added to wild-type Smad3 were created (Sm3+L1, Sm3+L2). In addition, to examine whether FSHβ activation by Smad3 requires direct DNA contact, the DNA-binding motif was deleted (Sm3Δβ). As indicated before, it was

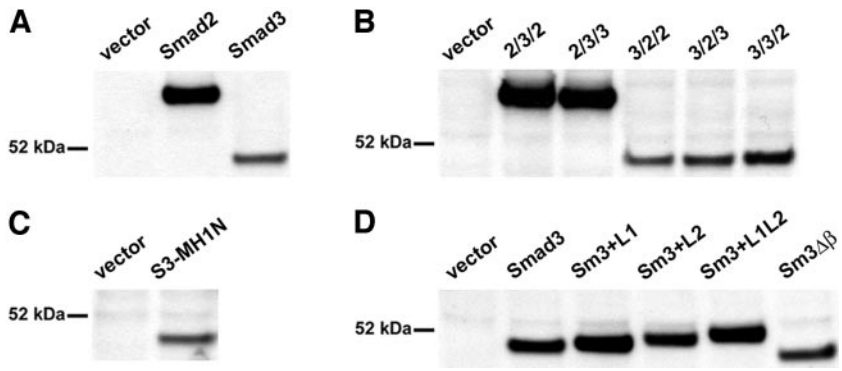

Fig. 3. Wild-Type, Mutant, and Chimera Smad Constructs Are Expressed in LβT2 Cell Line

LβT2 cells were transfected with the indicated myc-tagged Smad2 and Smad3 wild-type (A), Smad2/Smad3 chimeric (B and C), and Smad3 loop mutant (D) expression vectors. Cells were lysed and cell lysates were run on a polyacrylamide gel. Immunoblot was done using a mouse antimyc monoclonal antibody and goat antimouse HRP-conjugated secondary antibody.

confirmed by an immunoblot analysis that all constructs are expressed in LβT2 cells (Fig. 3D). Insertion of the second Smad2-like loop, as well as deletion of the DNA-binding β-hairpin loop, significantly hinder Smad3-mediated transactivation of the rat FSHβ promoter (Fig. 6). Contrary to the wild-type Smad3 or Smad3+L1, there is no synergistic effect of activin or Smad4 plus activin with either Sm3+L2 or Sm3Δβ mutant constructs.

DISCUSSION

Activin stimulates the transcription of target genes through the intracellular signaling molecules known as Smad proteins. Because activin is a major regulator of the FSHβ-subunit gene transcription, it is not surprising that Smad-mediated stimulation of the FSHβ subunit has been a subject of a number of recent studies. We have previously established that overexpression of

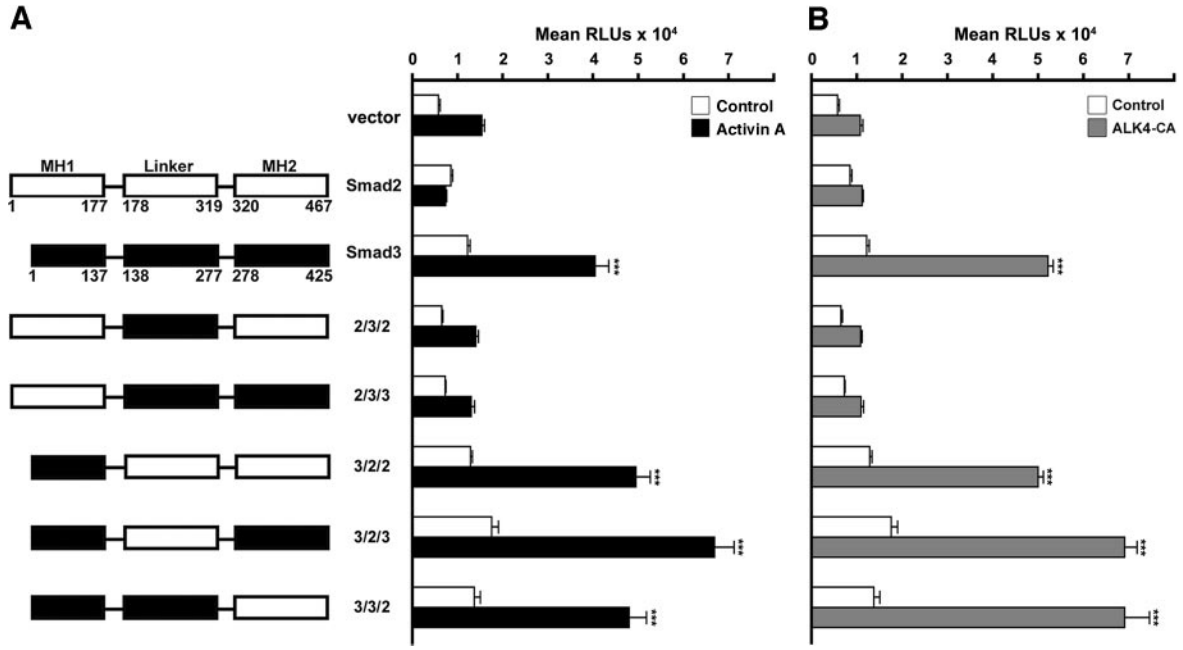

Fig. 4. The MH1 Domain of Smad3 Is Important for Activin- and ALK4-Mediated Stimulation of the Rat FSHβ Promoter

LβT2 cells were transiently transfected with the −338rFSHβ-Luc promoter construct and the indicated Smad wild-type or Smad2/Smad3 chimeric expression vectors and treated with control media or activin (30 ng/ml) for 24 h (A) or cotransfected with the plasmid encoding constitutively active ALK4 (ALK4-CA) receptor (B). Expression of FSHβ is reported as RLUs and is plotted as the mean ± SEM of triplicates from a representative experiment. A statistically significant difference between vector and Smad-transfected samples that were ligand or ALK4-CA treated is denoted by *asterisks* (***, $P < 0.001$). Boundaries of Smad domains (MH1, MH2, and Linker) that were swapped between Smad2 and Smad3 are indicated.

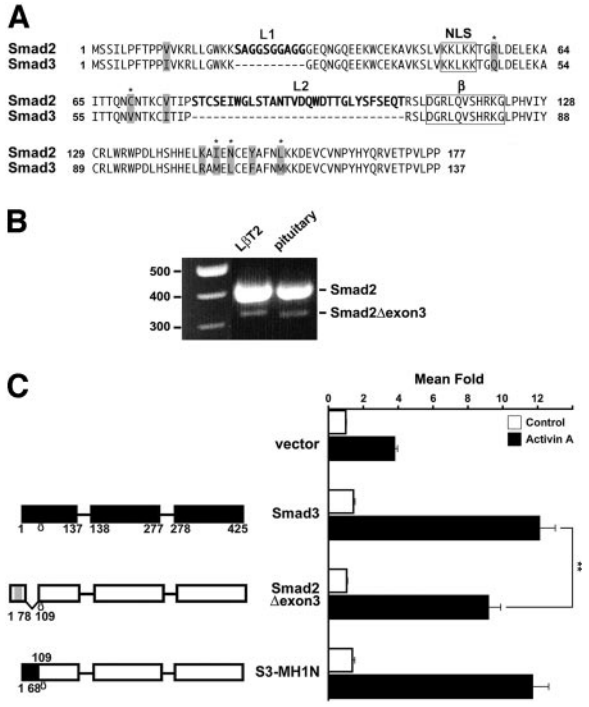

Fig. 5. The MH1 Domain of Smad2 and Smad3 Contains Significant Differences that Are Important for FSHβ Transactivation

A, Sequence alignment of mouse Smad2 and Smad3 proteins. Stretches of amino acids in Smad2 are *bold* and indicated as loop1 (L1) and loop 2 (L2); single amino acid differences are *shaded* and nonconserved residue changes indicated by *asterisks*; the DNA-binding β-hairpin loop sequence (β) and nuclear localization signal (NLS) are *boxed*. B, LβT2 and mouse pituitary mRNA were reversely transcribed to cDNA. Specific primer pairs were used to amplify cDNA of a wild-type and exon 3 deleted mutant (Smad2Δexon3) Smad2. PCR products (411 and 321 bp) were resolved on a 2% agarose gel. Negative control with no reverse transcriptase enzyme was also run (data not shown). C, LβT2 cells were transiently transfected with the −338rFSHβ-Luc promoter construct and cotransfected with the indicated Smad wild-type or mutant expression vectors, then treated with control media or activin (30 ng/ml) for 24 h. Data are plotted as the mean fold increase in Smad-transfected and ligand-stimulated promoter activity over activin-stimulated empty vector ± SEM of five independent experiments, each performed in quadruplicate. A statistically significant difference between samples transfected with Smad3 and Smad2Δexon3 and treated with activin is denoted by *asterisks* (**, $P < 0.01$). A schematic of wild-type and mutant Smads is presented. Smad2 loop (L1) is shown in *gray*, and β-hairpin motif is indicated.

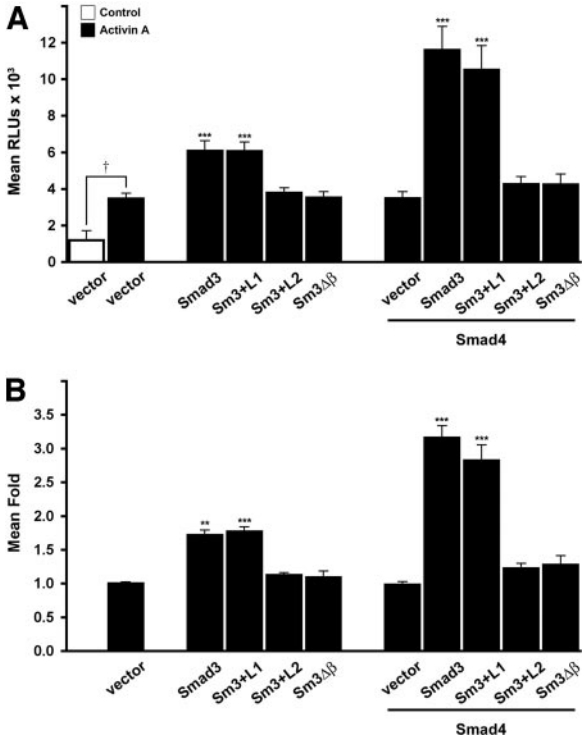

Fig. 6. The DNA-Binding Domain of Smad3 Is Important for Activin-Mediated FSHβ Transactivation

LβT2 cells were transiently transfected with the −338rFSHβ-Luc promoter construct and cotransfected with the indicated Smad wild-type or loop mutant expression vectors, then treated with control media or activin (30 ng/ml) for 24 h. A, Expression of FSHβ is reported as RLUs and plotted as the mean ± SEM of five independent experiments, each performed in quadruplicate. †, A statistically significant difference, as determined by the Student's *t* test, between control and activin-treated empty vector samples ($P < 0.001$). A statistically significant difference within activin-treated samples is denoted by *asterisks* (***, $P < 0.001$). B, Data are plotted as the mean fold increase in Smad-transfected and ligand-stimulated promoter activity over activin-stimulated empty vector ± SEM of five independent experiments, each performed in quadruplicate. A statistically significant difference between vector and Smad-transfected samples is denoted by *asterisks* (**, $P < 0.01$; ***, $P < 0.001$).

Smad3, but not Smad2, potently induces rat FSHβ promoter. This stimulation is highly augmented by a common mediator Smad4 as well as activin treatment (10). Although these results have been confirmed by others (13), a controversy exists regarding the exact mechanism by which activin-specific Smad proteins affect FSHβ promoter activity. Present data demonstrate that Smad3 is not only sufficient, but also necessary for FSHβ gene transcription, as a siRNA-mediated down-regulation of Smad3 protein expression abrogates activin stimulation of the FSHβ promoter. Interestingly, a small but significant decrease in activin-stimulated promoter activity was also observed with Smad2 siRNA. However, this was coupled to a decrease in basal transcription of FSHβ gene, suggesting that Smad2 may be important for basal regulation of the FSHβ promoter.

One of the advantages of our cell-based system is that the rat FSHβ promoter is stably transfected. Therefore, the exogenous rat FSHβ is exposed to the transcriptional machinery and has an established level of basal transcription before any attenuation of Smad protein expression occurs. Because LβT2 cells produce endogenous activin B, which can affect basal

FSHβ transcription (24), these cells provide a good model of FSH regulation that occurs *in vivo* systems, in which tonic levels of FSH are present. In our hands, delivery of specific siRNA shows selective ablation of each endogenous Smad protein. Moreover, oligoduplexes directed against Smad3, but not Smad2, abrogate activin-mediated stimulation of FSHβ gene transcription.

RNA interference technology has been used previously to study the role of Smad proteins in FSHβ transactivation (23). Bernard indicates that it is possible that Smad2 regulates basal transcription and Smad3 is important to activin-stimulated FSHβ activity, which is consistent with our findings. In our hands, fold induction by activin was significantly affected only by depletion of Smad3, and not Smad2, protein. Similarly, Gregory *et al.* (13) demonstrate that the active transcriptional complex that is responsive to activin and GnRH contains a DNA-binding Smad3 protein and not the scaffolding coregulator Smad2.

The report by Bernard also suggests that degradation of exogenous Smad2 transcripts in LβT2 cells prevents thorough investigation of this factor's role in activin-mediated stimulation of the FSHβ-subunit (23). We have demonstrated that Smad2 is expressed, activated and functional in LβT2 cells. Overexpression of Smad2 augments both activin- and TGFβ-mediated stimulation of the p3TP promoter construct, but it does not activate the rat FSHβ promoter in the same cell line. To propagate a signal from the cytoplasm to the nucleus, Smad proteins must be activated by a specific ligand. This activation results in Smad phosphorylation and subsequent translocation into the nucleus. Indeed, in LβT2 cell line Smad2 is phosphorylated and translocated into the nucleus upon activin treatment. The question that remains is why Smad2 is unable to induce transcription of the FSHβ gene.

Proteins of the Smad family have characteristic structural features that provide for the different functions of the molecule. Most Smads have two conserved domains at their amino and carboxy termini, termed MH1 and MH2, respectively, which are separated by a proline-rich linker region. The MH1 domain is important for both DNA binding and Smad-cofactor interactions, whereas the MH2 domain is essential for receptor-Smad, Smad-Smad and Smad-cofactor interactions (reviewed in Refs. 6, 7, 14, and 25). The collective results of the Smad2/3 chimera transfection experiments suggest that structural differences within the MH1 domain are important for Smad3-mediated stimulation of the FSHβ-subunit. The chimera constructs containing the MH1 domain of Smad2 were unable to transactivate the FSHβ promoter independent of the MH2 domain or the linker region.

The ability of Smad factors to bind DNA directly is conferred through a highly conserved 11-residue β-hairpin in the MH1 domain (19). This DNA-binding motif was found to be necessary for activation of the FSHβ promoter because the Smad3 mutant lacking this sequence was unable to stimulate activin-mediated FSHβ transcription. Although Smad-DNA interaction is of low affinity, DNA binding of transcriptional cofactors has been shown to be important for the transcriptional activation of specific target genes. In fact, a Smad-binding consensus sequence capable of Smad3 and Smad4 interaction was found in the proximal rat FSHβ promoter (10, 13). Furthermore, this binding motif was shown to be necessary for Smad- and activin-mediated stimulation of FSHβ promoter activity. Therefore, the failure of Smad2 to transactivate the FSHβ promoter may result from its inability to bind directly to DNA. Activin-mediated stimulation of the FSHβ-subunit may require direct Smad-DNA interaction, whereas other promoters, such as p3TP, may be transactivated by Smad2 indirectly, via contact with other Smads or transcription factors.

Although both Smad2 and Smad3 contain the β-hairpin motif, a direct interaction of the Smad2 protein with DNA has not been reported. It has been suggested that a stretch of 30 amino acids encoded by exon 3 and located just upstream of the β-hairpin motif is responsible for the inability of Smad2 to bind to DNA (18, 26). Indeed, insertion of the Smad2 L2 sequence into the MH1 domain of Smad3 completely abrogates activin-mediated stimulation of the FSHβ promoter. This further confirms that the Smad2 loop regions, specifically L2, hinder the ability of Smad2 to transactivate the rat FSHβ promoter. Interestingly, the Smad2 splice variant that lacks exon 3 (Smad2Δexon3) and the interfering L2 sequence is present in both pituitary and LβT2 cell line. Similarly to previous report (23), overexpression of this factor can induce FSHβ promoter activity. Although these data may suggest that Smad2Δexon3 could play a role in activin-mediated FSHβ gene transcription *in vivo*, this stimulation is not as robust as wild-type Smad3. In addition, in our siRNA studies we have employed a pool of short interfering oligoduplexes to achieve complete ablation of each Smad protein. Our approach permits us to eliminate both Smad2 and Smad2Δexon3 to ensure that the small contribution of the alternatively spliced Smad2 isoform is also eliminated. Thus, although it is possible that both Smad2 and Smad2Δexon3 are involved in basal FSHβ transcription, it is unlikely that these molecules are involved in activin-mediated FSHβ gene regulation in the pituitary.

Functional properties of Smad2Δexon3 have been recently examined in the mouse embryo (27). Dunn *et al.* used a genetic complementation to show that overexpression of this isoform can restore majority of functions lost in Smad2-deficient embryonic stem cells and embryos. The authors also suggest that there is a significantly higher abundance of the alternatively spliced Smad2 variant transcripts during different developmental stages and in various adult tissues than previously reported (26, 28). This indicates that Smad2Δexon3 may in fact play an important role as a downstream modulator of TGFβ/activin pathway. These conclusions, however, are based on Smad functions in the early embryo. We, and others (23),

have shown that abundance of Smad2Δexon3 transcripts in an adult mouse pituitary and LβT2 cell line is significantly lower that that of full-length Smad2. In addition, this factor cannot fully mimic transcriptional activity of Smad3 in regards to the rat FSHβ promoter activation, and its silencing has no significant effect on activin-mediated stimulation of FSHβ gene. It is possible that structural differences within the receptor-regulated Smad proteins account for cell and tissue specificity of TGFβ/activin action as well as differential regulation of target gene expression. Further analysis of differential expression and functions of these molecules is necessary to fully understand their diverse, and often opposing, actions.

Although only ligand-activated Smad complexes can induce a transduction cascade, nucleoplasmic shuttling of the Smad proteins occurs continuously. The Smads can enter the nucleus in an uninduced state and their ability to do so may provide a level of distinctive intracellular control of the signal (29). A number of structurally based nuclear import mechanisms have been implicated in Smad shuttling. Smad2, for example, can directly interact with the nucleoporins CAN/Nup214 and Nup153 through its MH2 domain and this step is inhibited by an interaction of Smad2 with the Smad anchor for receptor activation (SARA) protein (30, 31). Interestingly, a nuclear localization signal in the MH1 domain of Smad3 has been shown to mediate nuclear import through a direct interaction with importin-β (32). The presence of the exon 3-encoded insertion that prevents Smad2 from direct DNA binding may also prevent its interaction with importin-β (33). In the basal state, endogenous Smad3 is found in large complexes with other proteins, whereas Smad2 is primarily monomeric (34). Because the cyclical pattern of FSH action requires a highly regulated and rapid stimulation, it is possible that pre-bound Smad3 complexes exist in the LβT2 cytoplasm in an uninduced state, ready for movement into the nucleus upon activin stimulation. In fact, Smad3 was found to mediate transcriptional repression of the c-*myc* gene through a preexisting complex containing Smad3 and specific transcription factors (35).

As a member of the TGFβ superfamily, activin controls diverse cellular responses including differentiation, proliferation, controlled cell death, and migration. Although activin affects the expression of many genes, activin-regulated FSH biosynthesis is exclusive to pituitary gonadotropes. Specificity of cellular response is often controlled by tissue- and cell-specific transcription factors. Pituitary-specific members of the *bicoid*-related homeodomain proteins, Pitx1 and Pitx2, were recently identified as transcriptional coregulators that permit cell type-restricted FSH response (10, 36). The ability to bind specific transcription factors is dictated by different domains of the Smad proteins. For example, Smad2-FAST1 interaction is mediated via a region known as αH-2 in the MH2 domain, whereas Smad3 binds FAST2 through its MH1 domain (37, 38). Therefore, it is possible that structural features of Smad2 and Smad3 that underlie their binding to a pituitary cofactors are also important for the differences between these two proteins in terms of their ability to regulate FSHβ gene activity.

In conclusion, the results of these studies indicate that the functional differences between the ability of Smad2 and Smad3 to transactivate the rat FSHβ promoter lie primarily within the MH1 domain and involve structural motifs that affect DNA binding and/or protein-protein interactions. Whereas Smad2 and/or Smad2Δexon3 might be involved in basal transcription of FSHβ, Smad3 is not only sufficient but also necessary for activin-mediated regulation of the FSHβ promoter. The ability of Smad3 to transactivate the promoter requires direct DNA binding through the β-hairpin motif. Moreover, interactions of Smad3 with a pituitary-specific factor likely exist and further mapping of the Smad3 regions responsible for FSHβ gene transcription will provide further insight into the mechanisms of activin-stimulated target gene specificity in the pituitary.

MATERIALS AND METHODS

Recombinant Ligands

Recombinant human activin A (activin) was produced in our laboratory and formulated in a buffer of 0.15 M NaCl and 0.05 M Tris (pH 7.5) (39).

Cell Culture and Transient Transfections

The pituitary gonadotrope cell line LβT2 (40) was carried on plates coated with matrigel (BD Biosciences, Bedford, MA) in F12:DMEM supplemented with 5% fetal bovine serum (FBS), 0.45% glucose and 1% antibiotic in a humidified atmosphere (37 C) of 5% CO_2 and passaged as necessary. All transfections and experimental treatments were described before (10). Briefly, cells were plated 1 d before transfection in 24-well plates and transfected with 250 ng of the reporter DNA and 25 ng of the expression vector per well using LipoFectamine Plus or LipoFectamine 2000 (Invitrogen, Carlsbad, CA). Empty vectors were used to balance DNA where necessary. Cells were treated with control media or activin (30 ng/ml) for 24 h in phenol-free, serum-free F12:DMEM (Invitrogen). We attempted to use internal controls for all transfection experiments by dual luciferase and β-galactosidase assays. Unfortunately, cotransfection of both *Renilla* luciferase and β-galactosidase expression vectors caused a significant decrease in activin response. Additionally, LβT2 cells were grown on a matrigel matrix, which interfered with normalization of luciferase activity to protein content. The data shown here reflect the actual relative light units and are representative of the mean and SEM of at least three separate transfection experiments.

LβT2-F338 cell line was created by cotransfecting the LβT2 cells with a plasmid containing 338 bp of the 5′-flanking region of rat FSHβ gene fused to the luciferase reporter gene (−338rFSHβ-Luc) and an empty pcDNA3 vector. Cells were then treated with increasing doses of G418 sulfate (Cellgro, Herndon, VA) until a stable population of cells was established and maintained in normal growth media containing 1 mg/ml of G418 reagent. Cells were grown and passaged as described above for the LβT2 cell line.

DNA Constructs

The reporter construct consisting of 338 bp of the 5′-flanking region of rat FSHβ gene fused to the luciferase reporter gene (−338rFSHβ-Luc) was described previously (10). Wild-type Smad plasmids and the cloning strategy used to generate Smad2/Smad3 chimera expression vectors were described previously (38). Smad3 loop and β-hairpin mutants were made using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and Smad2Δexon3 was the generous gift of Dr. M. Kato (The Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan) and was described previously (26). Sequences of all constructs were confirmed.

Smad2 (M-004355-00-05), nonspecific control (D-001206-09-05) and custom-designed Smad3 siRNA oligoduplexes were purchase from Dharmacon, Inc. (Lafayette, CO). Transfection and luciferase assay of the siRNA duplexes into LβT2-F338 cells was performed as described above for transient transfections of cDNA expression vectors into LβT2 cells.

RT-PCR Analysis

Total RNA from LβT2 cells and mouse pituitaries was isolated using the TRIzol reagent (Invitrogen) and samples were treated with RQ1 ribonuclease-free deoxyribonuclease (Promega, Madison, WI) and phenol-chloroform extracted. RNA samples (5 μg) were then primed with random hexamers and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) according to manufacturer's instructions. The cDNA from the original rt reaction was subjected to PCR amplification for 35 cycles under the following conditions: 94 C for 60 sec, 54 C for 60 sec, 72 C for 60 sec. Negative controls were run using water and RNA that had not been reverse transcribed with Moloney murine leukemia virus. The full-length Smad2 (411 bp) and Smad2Δexon3 (321 bp) were amplified using following primer set: forward 5′-ATCTTGCCATTCACTCCGCC-3′ and reverse 5′-ATGACT-GTGAAGGTCCGGCC-3′. Amplified products were resolved on 2% agarose gel.

Immunoblot Analysis

LβT2 cells were plated in six-well plates and transfected with the indicated empty or Smad expression vectors. Cells were lysed in a buffer containing 50 mM Tris (pH 7.5), 10% glycerol, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40. Lysates were run on NuPage 4–12% Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose. Immunoblot analysis was performed using mouse monoclonal antimyc antibody (Sigma-Aldrich, St. Louis, MO) and goat antimouse horseradish peroxidase (HRP)-conjugated secondary antibody (Zymed, South San Francisco, CA). For siRNA immunoblot analysis lysates from cells adjacent to those used for a luciferase assay were run and transferred as described above. Rabbit polyclonal anti-Smad2 and anti-Smad3 antibodies (Zymed) and donkey antirabbit HRP-conjugated secondary antibody (Amersham Biosciences, Inc., Piscataway, NJ) were used to detect levels of endogenous Smad2 and Smad3 protein levels. Immunoblot results were visualized using an ECL detection reagent (Amersham Biosciences, Inc.)

Immunofluorescence

LβT2 cells were plated on matrigel-coated chamber slides 1 d before treatment with 30 ng/ml activin for 15, 30, and 60 min. Cells were washed twice in PBS, fixed for 10 min in 4% paraformaldehyde, washed twice in PBS, and permeabilized for 2 min with ice cold 100% methanol. Cells were then washed in PBS and incubated in 10% normal donkey serum (Jackson ImmunoResearch, Inc., West Grove, PA) for 1 h at room temperature, followed by incubation with rabbit antiphoshoSmad2 antiserum (courtesy of Dr. Peter ten Dijke, the Netherlands Cancer Institute, Amsterdam, The Netherlands) diluted 1:2000 in 10% normal donkey serum overnight at 4 C. After four washes in PBS, cells were incubated in fluorescein isothiocyanate (FITC)-conjugated donkey antirabbit antibody (Jackson ImmunoResearch, Inc.) diluted 1:200 in 10% normal donkey serum for 1 h at room temperature. Slides were washed twice in 1× PBS, briefly dried, mounted with Vectastain with DAPI (Vector Laboratories, Inc., Burlingame, CA) and sealed. Slides were viewed using fluorescence microscopy and digital images were collected using a SpotRT monochrome digital camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed using the Metamorph image analysis system (version 4.5; Universal Imaging Corp., West Chester, PA).

Statistical Analysis

The values are expressed either as the relative light units (RLUs) or mean fold ± SEM. ANOVA followed by Tukey's *post hoc* test was used to evaluate differences between treatment groups as indicated in the figure legend. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) were considered statistically significant. Student's *t* test was used to evaluate a difference between the control and activin-treated empty vector samples in Fig. 6A (†, $P < 0.001$).

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